



# Immunization with a DNA vaccine cocktail induces a Th1 response and protects mice against *Mycobacterium avium* subsp. *paratuberculosis* challenge

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## ABSTRACT

Several antigens of *Mycobacterium avium* subsp. *paratuberculosis* have been studied as vaccine components and their immunogenicity has been evaluated. Previously, we reported that 85 antigen complex (85A, 85B, and 85C), superoxide dismutase (SOD), and 35 kDa protein could induce significant lymphocyte proliferation as well as the elaboration of Th1-associated cytokines including interferon gamma (IFN- $\gamma$ ), interleukin-2 (IL-2), IL-12 and tumor necrosis factor alpha (TNF- $\alpha$ ). Based on these results, we cloned and expressed 85A, 85B, 85C, SOD, and 35 kDa-protein genes into the eukaryotic expression plasmid pVR1020. C57BL/6 mice were immunized three times intramuscularly with the recombinant DNA cocktail and pVR1020 DNA alone as control. A significant reduction in the bacterial burden in the spleen and liver of mice immunized with the DNA cocktail as compared to the vector control group was found. Also, the relative severity of the liver and spleen histopathology paralleled the MAP culture results, more granulomas and acid-fast bacilli in the vector control animals. Moreover, mice immunized with the DNA cocktail developed both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to the recombinant antigens and showed significant lymphocyte proliferation. The Th1 response related cytokine (IFN- $\gamma$ ) levels increased in splenocytes obtained from immunized animals. These results indicate that the use of a recombinant DNA vaccine can provide protective immunity against mycobacterial infection by inducing a Th1 response.

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## 1. Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD), a chronic granulomatous enteritis of domestic and wild ruminants [1,2]. Clinically affected cattle develop chronic diarrhea and progressive weight loss 2–5 years after infection that eventually results in death. Subclinically infected animals mainly have decreased milk production. In North America, MAP infection is widespread in dairy herds. Early studies, based on culture of ileocecal lymph nodes collected at slaughter, estimated the prevalence in culled dairy cows to be 2.9% [3]. The 1996 USDA National Animal Health Monitoring System's study estimated a herd prevalence of 22% based on serology and clinical history with a herd prevalence rate of 40% in larger herds [4]. The economic losses of this disease are estimated to cost the US dairy

industry \$200–\$250 million USD annually [5]. In addition to direct economic losses, premature culling of infected animals reduces the herd manager's ability to cull for other reasons such as low productivity or other health problems and can result in the loss of valuable genetic potential [6].

Although vaccination with either heat-killed or live attenuated MAP can reduce the level of bacterial shedding in the feces and decrease the severity of clinical disease [7–19], the protection offered is not complete. A recent report indicates that vaccination of calves with a killed vaccine does not prevent transmission of MAP [10], and the current consensus about the available MAP vaccines is that they do not provide complete protection [20,23,28,40]. Thus, development of a potent vaccine that stimulates protective immunity but that does not interfere with tuberculosis control programs is a current research priority.

DNA vaccines encoding *M. tuberculosis* antigens have been studied in animal models [20–24]. DNA vaccines encoding *M. tuberculosis* proteins like 85A and 85B induce partial protection against experimental tuberculosis [25,26]. More relevant to the current study, DNA vaccination was previously shown to protect against *M. avium* subsp. *paratuberculosis* challenge in BALB/c mice [27]. Pre-

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viously, we reported that five recombinant antigens, which include 85 antigen complex (85A, 85B, 85C), superoxide dismutase (SOD), and 35 kDa protein, could induce significant lymphocyte proliferation as well as the secretion of Th1-associated cytokines including interferon gamma (IFN- $\gamma$ ), interleukin-2 (IL-2), IL-12, and tumor necrosis factor alpha (TNF- $\alpha$ ) [28]. We have also found that these antigens used as a recombinant protein vaccine can induce protection against challenge in calves [29]. In light of this background, we are interested in developing a DNA vaccine cocktail against MAP infection. We have cloned the 85 antigen complex (85A, 85B, 85C), SOD and 35 kDa protein genes of MAP in the mammalian expression vector, pVR1020 and studied the protective efficacy of these antigens as a DNA cocktail against MAP challenge in a mouse model.

## 2. Materials and methods

### 2.1. Animals

Eight-week-old C57BL/6 female mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The animals were maintained in a biosafety level II facility with free access to feed and water. All of the experimental work was conducted in compliance with the regulations, policies, and principles of the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals used in Testing, Research, and Training, the NIH Guide for the Care and Use of Laboratory Animals and the New York State Department of Public Health.

### 2.2. DNA vaccine construction and expression in mammalian cells

We used the eukaryotic expression plasmid pVR1020 (Vical, Inc., San Diego, CA) for cloning and expression of DNA vaccine constructs. This plasmid contains an immediate-early cytomegalovirus promoter to ensure efficient expression in eukaryotic hosts, as well as the human tissue plasminogen activator (hTPA) secretion signal to facilitate secretion of the target antigen from the eukaryotic cell [30]. MAP specific 85A, 85B, 85C, SOD, and 35 kDa genes were amplified by PCR using gene specific primers (Table 1), and each fragment was ligated into pCR2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA). The *Bam*HI/*Bgl*II fragment from each of these five constructs was then ligated into pVR1020 as previously described [28] and subjected to DNA sequencing. Each DNA vaccine construct was prepared with a Mega-prep system, which is commercially available (Qiagen Plasmid Mega kit, USA). Each of the five DNA vaccine constructs were transfected into HEK-293 (human embryonic kidney) cells using lipofectamin<sup>TM</sup> 2000 reagent (Invitrogen) and expression was checked at the transcript level using reverse transcriptase PCR (RT-PCR) and western-blot analysis (data not shown).

### 2.3. Immunization and challenge

Mice were divided into two groups each comprising 28 animals (18 for challenge studies and 10 for immunological studies). Group I animals received the DNA cocktail (85A, 85B, 85C, SOD, and 35 kDa) gene constructs. Group II animals were kept as control animals and received the vector DNA (pVR1020). The animals were given 50  $\mu$ g of each DNA in 50  $\mu$ l PBS intramuscularly, three times at 3-week intervals. Three weeks after the second booster, 18 animals in each group were challenged by intraperitoneal injection of 10<sup>9</sup> CFU of MAP, which were isolated from a calf diagnosed with JD. After challenge, feces were collected every week from mouse cages and were enumerated on Herald's Egg Yolk (HEY) slants supplemented with Mycobactin J and antibiotics as previously described [31]. Six animals in each group were sacrificed at 4, 8, and 12 weeks after

challenge and bacterial loads in the liver, spleen, mesenteric lymph node and lung were determined using the same culture medium as for the fecal cultures. In addition, sections of liver, spleen, lung, intestine and mesenteric lymph node were fixed by immersion in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 4  $\mu$ m and stained with hematoxylin and eosin using conventional histological methods and examined by light microscopy. The severity of the inflammatory response was assessed on the number and size of granulomas according to previously published methods with some modifications [32–34]. Briefly, the number and size of granulomas were counted in ten 40 $\times$  fields selected at random. Small granulomas (up to 25  $\mu$ m in diameter) consisted primarily of small lymphocytes that surrounded a few epithelioid macrophages. Large granulomas (up to 100  $\mu$ m in diameter) were surrounded by a thin fibrous capsule and were composed almost exclusively of epithelioid macrophages. The inflammatory response was ranked as: (0) normal, (1) mild inflammation (fewer than 1 small granuloma per 40 $\times$  field), (2) moderate inflammation (1–3 granulomas per 40 $\times$  field with a mixture of small and large granulomas) and (3) severe inflammation (>3 large granulomas per 40 $\times$  field).

The presence of acid-fast bacteria in the tissues was assessed by Ziehl–Neelsen staining. The experiment was repeated twice with the same number of animals each time.

### 2.4. Recombinant antigen (rAg) preparation

Recombinant MAP antigens (rAgs) 85A, 85B, 85C, 35 kDa, and SOD, were cloned, expressed and purified as previously described [28]. The expressed proteins were purified using Ni-NTA agarose columns (Qiagen, Valencia, CA). Endotoxin contamination was removed by using Affinity Pak Detoxi Gel (Pierce, Rockford, IL). Purified antigens were used for *in vitro* stimulation of spleen cells for immunological assays. The antigens used in this study had negligible (10 pg/ml) endotoxin as determined in a Limulus amoebocyte assay (MP Biomedicals, USA).

### 2.5. Spleen cell culture for immunological assays

Four weeks after the second boost, 10 mice from each group were sacrificed and spleen cells were obtained by conventional methods. Briefly, spleens were removed aseptically, pressed through a sterile Falcon cell strainer using a plunger and the red cells were lysed with Tris–NH<sub>4</sub>Cl buffer (0.75% NH<sub>4</sub>Cl and 0.205% KHCO<sub>3</sub> in dH<sub>2</sub>O, pH 7.2). After resuspending in RPMI 1640 medium (10% FBS, 50  $\mu$ M 2- $\beta$ -ME, 1% penicillin/streptomycin, 0.1% gentamicin), cells were plated at a final density of 10<sup>6</sup> cells/well in 96-well Falcon flat or round bottom plates depending on the type of assay used.

### 2.6. Lymphocyte proliferation assay

After stimulating the cells with rAgs, lymphocyte proliferation was measured by Cell proliferation ELISA, BrdU colorimetric kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. Results were read in an ELX 808 Ultra microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) and expressed as a stimulation index (SI), calculated as the ratio between the mean OD of cells cultured with the antigens and the mean OD of cells cultured without the antigens.

### 2.7. Flow cytometric analysis

Spleen cells were cultured in duplicate in round bottom 96-well tissue culture plates with 1  $\times$  10<sup>6</sup> cells/well for 24 h. After stimulating with each rAg or ConA for an additional 24 h, FACS analysis was

**Table 1**

Details of primer sequences used for cloning into pVR1020

Gene and primers	Sequence (5' → 3')	Length	Accession No.
85A (MAP0216) pVR85AF pVR85AR	CGGGATCCATGATGACGTTGTCGACA CGGGATCCTTAGGTGCCTGG	1050 bp	AF280067
85B (MAP1609c) pVR85BF pVR85BR	CGGGATCCATGACAGATCTG CGGGATCCTTATCCGCCGCC	1000 bp	AF219121
85C (MAP3531c) pVR85CF pVR85CR	CGGGATCCATGTCGTTTCATCGAA CGGGATCCTCAGGTGGCGGGC	1100 bp	AF280068
SOD (MAP0187c) pVRSODF pVRSODR	GGATCCTGGGACTATGCAGC AGATCTTCAGCCGAAGATCAGGC	590 bp	AF180816
35 KDa (MAP2121c) pVR35KDF pVR35KDR	GGATCCCCACTTGGTGATCT AGATCTTCACCTGTACTCATGGAAC	910 bp	AF232751

performed with standard procedures. Briefly, cells were washed three times with FACS buffer (1% BSA and 0.05% sodium azide in PBS) and resuspended in 50 µl of FACS buffer with the same volume (0.5 µg/50 µl) of FITC or PE conjugated CD4(CD4[RM4-5;FITC]) and CD8(CD8a[53–6.7;PE]) antibodies (eBioscience, San Diego, CA), and incubated on ice for 30 min. Cells were washed twice with FACS buffer and suspended in 100 µl of 3% formaldehyde in PBS and transferred to FACS tubes containing 500 µl of PBS. Data were collected on 10,000 events using a FACS caliber flowcytometer (Becton-Dickinson, San Jose, CA) and analyzed using Cellquest software. The results were expressed as the average percent of induced cells with positive staining relative to that of the uninduced sample stained with the same antibody.

## 2.8. ELISPOT assay

In order to determine the relative number of IFN-γ expressing cells in the single cell suspensions, we performed ELISPOT assay. A 96-well filtration plate (KPL®) was coated with 10 µl/ml of rat anti-mouse IFN-γ capture Ab (BD Pharmingen, San Jose, CA) and incubated overnight at 4 °C. After washing with PBS three times, the plates were blocked with 10% FBS for 1 h at room temperature. Spleen cells were plated in duplicate from  $1 \times 10^5$  cells/well in 100 µl to  $1 \times 10^3$  cells/well by serial dilution and incubated at 37 °C for 24 h in a humidified atmosphere supplemented with 5% CO<sub>2</sub>. rAgs (10 µg/ml) or ConA (10 µg/ml) were used to stimulate the cells for 48 h at 37 °C with 5% CO<sub>2</sub>. The plates were washed with PBS and incubated overnight with biotin-conjugated, rat anti-mouse IFN-γ secondary

antibody. After incubation, filters were developed with avidin-peroxidase conjugate and substrate kit (KPL, Gaithersburg, MD). The plates were dried in the dark, and the spots were counted by microscopy.

## 2.9. Real-time quantitative RT-PCR for cytokine assays

From each vaccinated group, spleen tissue was collected to evaluate cytokine gene expression profiles. Total RNA isolation, reverse transcription, and real-time quantitative RT-PCR were performed as described earlier [32]. Frozen tissues were homogenized and lysed by conventional methods and RNA was isolated using an RNeasy mini Kit (Qiagen, Valencia, CA). The isolated RNA samples were treated with 10 U/µl of RNase free DNase I (Qiagen, Valencia, CA) at 37 °C for 10 min, followed by heat inactivation at 95 °C for 5 min and then chilled on ice. The primer and probe sets (Table 2) used in this study were as previously described [32]. The probes were labeled with the fluorescent reporter dye, 6-carboxyfluorescein (FAM) at the 5' end and the quencher dye, N', N',N',N'-6-carboxytetramethylrhodamine (TAMRA) at the 3' end. PCR reaction was performed in 25 µl volumes containing 2 µl of 10 pM forward and reverse primers, 2 µl of 2 pM probe, 12.5 µl of TaqMan PCR master Mix and 9.5 µl of diluted cDNA using following conditions: 10 min at 94 °C, followed by a total of 40 two-temperature cycles (15 s at 95 °C and 1 min at 60 °C), in an automated fluorometer (7700 Sequence detector, Applied Biosystems, Foster city, CA). Quantitation was done using the comparative cycle threshold (C<sub>T</sub>) method and reported as relative transcription or the *n*-fold difference relative to a calibrator cDNA.

**Table 2**

Sequences of PCR primers and TaqMan probes specific for murine cytokines

Cytokine	Primer & probe sequence (5' → 3')	Length (bp)	Accession No. <sup>a</sup>
IFN-4	F: AGA TGGATGTGCCAAACGTCCTCA R: AATATGCGAAGCACCTTGGGAAGCC P: AAGAACACCACAGAGAGTGAGCTCGT	88	NM.021283
IFN-10	F: TGCACTACCAAAGCCACAAAGCAG R: AGTAAGAGCAGGCAGCATAGCAGT P: AAAGAGAGCTCCATCATGCCTGGCT	86	NM.010548
IFN-γ	F: TCAAGTGGCATAGATGTGGAAGAA R: TGGCTCTGCAGGATTTTCATG P: TCACCATCCTTTTGCCAGTTCCTCCAG	92	M28381
GAPDH	F: TCACCACCATGGAGAAGGC R: GCTAAGCAGTTGGTGGTGCA P: ATGCCCCCATGTTTGATGGGTGT	168	U09964

F, forward primer; R, reverse primer; P, TaqMan probe, dual-labeled with 5' FAM and 3' TAMRA.

<sup>a</sup> Genebank accession number of cDNA and corresponding gene, available at <http://www.ncbi.nlm.nih.gov/>.

### 2.10. IgG isotype ELISA

Mice were bled periodically (before vaccination, 1st booster, 2nd booster, and 4 weeks after final booster), and serum IgG isotype response was measured by conventional ELISA. ELISA plates (Nunc-immuno module, Nunc, Roskilde, Denmark) were coated with 200 ng/well of rAgs and incubated at 4 °C overnight. After washing once with 0.05% Tween 20 in PBS (PBST), 300 µl of blocking buffer (1% BSA in PBST) was added and incubated at 25 °C for 1 h. Plates were washed thrice with PBST and 100 µl of diluted serum samples were added to the wells and incubated at 37 °C for 1 h. After washing, 25 ng of biotin-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotech, Birmingham, AL) was added to the wells and incubated at 25 °C for 30 min. Following washing, 0.2 µg/ml of streptavidin labeled with horseradish peroxidase (KPL) was added and incubated at 25 °C for 30 min. After washing, 50 µl of 3,3', 5, 5'-tetramethylbenzidine was added to the wells and incubated for 15 min. Plates were read in an ELx 808 Ultra microplate reader by endpoint method at 450 nm after adding 50 µl of 1N H<sub>2</sub>SO<sub>4</sub> stop solution. Results were expressed as the ratio between the IgG1 and IgG2a OD values.

### 2.11. Statistical analysis

Microsoft Office Excel software was used to perform statistical analysis. Differences between groups, individual antigens and cytokine gene expression were analyzed with the Student's *t*-test. Differences were considered significant when a probability value of <0.05 was obtained.

## 3. Results

### 3.1. DNA vaccine induces lymphoproliferative responses in immunized mice

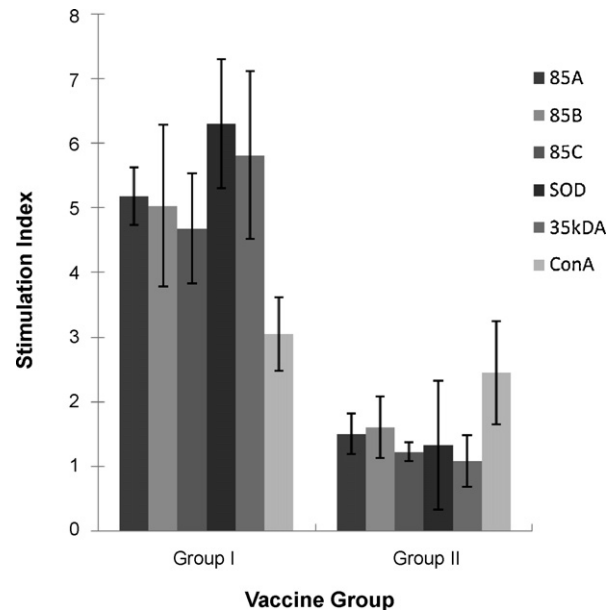
Group I mice immunized with a MAP DNA vaccine consisting of the five study genes showed a significant ( $P < 0.05$ ) increase in antigen specific lymphoproliferative responses when compared to the group II control animals (Fig. 1). The proliferative responses were ~4- to 6-fold higher in immunized animals.

### 3.2. Immunization with DNA cocktail alters antigen specific lymphocyte subset distribution

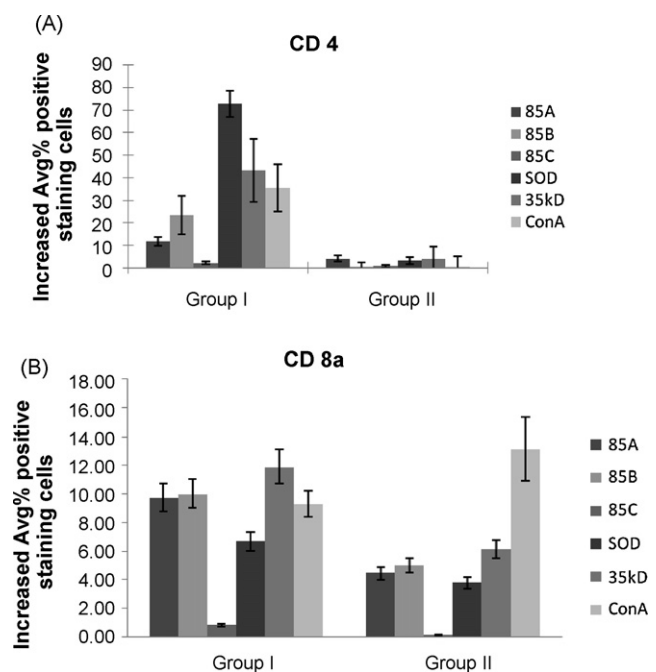
We also analyzed the antigen specific T-cell repertoire stimulated *in vivo* following immunization with the DNA cocktail. The proportion of antigen specific CD4<sup>+</sup> T cells was higher in the immunized group than the control group. 85B, SOD, and 35 kDa specific CD4<sup>+</sup> T cell populations were significantly higher ( $P < 0.05$ ) in immunized animals than the control animals (Fig. 2A). Furthermore, the CD8<sup>+</sup> T cells showed a significantly higher ( $P < 0.05$ ) response for 85A, 85B and 35 kDa antigens in the immunized animals in contrast to the control animals (Fig. 2B). Neither lymphocyte subset was stimulated by 85C.

### 3.3. DNA cocktail induces a strong Th1 response in immunized mice

Relative expression of various cytokine genes was assessed using real time PCR on mRNA isolated from spleen cells. Expression of IFN-γ was higher in group I animals than the control group II (Fig. 3). Overall, expression of the Th1 cytokine IFN-γ in the immunized animals was higher than the Th2 cytokines IL-4 and IL-10 ( $P < 0.05$ ). To further investigate the differences in IFN-γ response to

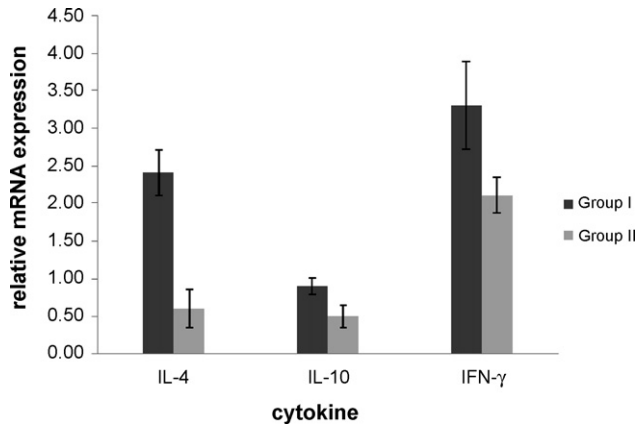


**Fig. 1.** Lymphoproliferative responses of splenocytes from immunized (group I) and control (group II) mice stimulated with rAgs (85A, 85B, 85C, SOD, and 35 kDa) and ConA (a positive control lectin). The results are expressed as stimulation index (SI), calculated as the ratio between the mean OD of cells cultured with the antigens and the mean OD of cells cultured without the antigens. Data represent means  $\pm$  standard errors (S.E.) from three independent experiments.



**Fig. 2.** Expression of lymphocyte subsets from splenocytes collected from immunized (group I) and control (group II) mice 4 weeks after the 2nd booster. After stimulating with rAgs (85A, 85B, 85C, 35 kDa, and SOD) or ConA for an additional 24 h, cells were incubated with FITC or PE conjugated (A) CD4(CD4[RM4-5;FITC]) and (B) CD8(CD8a[53-6.7;PE]) antibodies. Results are expressed as the fold increase of cells with positive staining relative to the un-induced (cultured with medium) cells stained with the same antibody. Data represent mean  $\pm$  S.E. from three independent experiments.



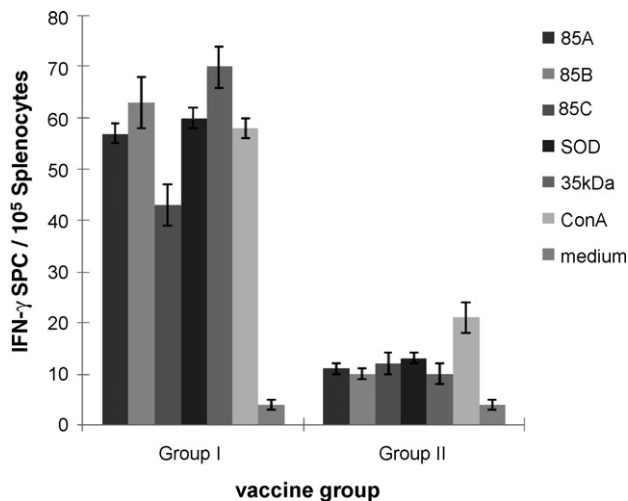


**Fig. 3.** Cytokine gene expression in response to recombinant and control antigens in immunized (group I) and control (group II) mice. Results are expressed as relative mRNA expression by calculating the ratio of each cytokine expression to that of a house keeping gene (GAPDH). Data represent mean  $\pm$  S.E. from three independent experiments.

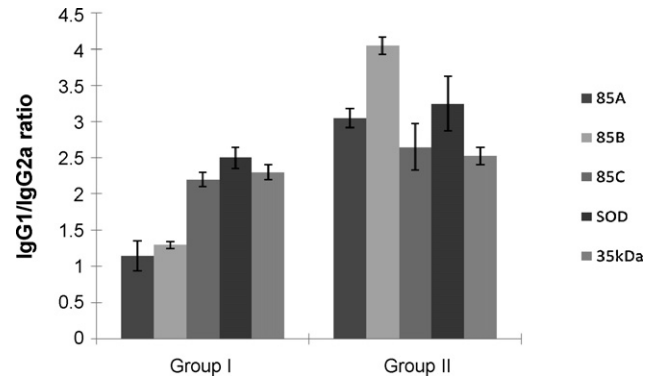
the DNA cocktail antigens, we performed an ELISPOT assay on each treatment group. As shown in Fig. 4, the number of IFN- $\gamma$  secreting cells was significantly higher in the immunized group than the control group after stimulating with the antigens. Together, these results indicated that the DNA vaccine strongly induced Th1-directed immune responses.

#### 3.4. MAP DNA vaccine enhances a cellular immune response

Sera from mice were tested for antigen specific IgG1 and IgG2a subclass profiles. The titers of IgG1 and IgG2a were significantly higher in immunized groups than the control group (data not shown). IgG1/IgG2a ratios were comparatively low in the immunized group compared to the control (Fig. 5). Especially, from sera at 4 weeks after final booster, IgG1/IgG2a ratios were two to threefold less in group I than II. Considering that IgG2a is more crucial for Th1 responses, the results indicate DNA vaccination induced a Th1 biased immune response.



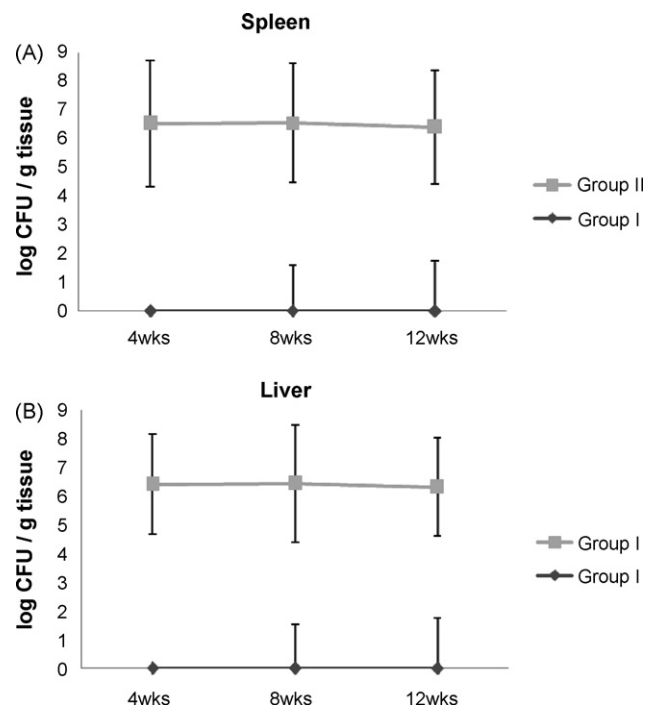
**Fig. 4.** Antigen-specific IFN- $\gamma$  responses by splenocytes from immunized (group I) and control (group II) mice. After immunization with the DNA cocktail or control vector DNA, the spleens were removed and the antigen-specific spot forming cells (SFCs) per  $10^5$  cells were determined by ELISPOT analysis. Data represent mean SFCs per  $10^5$  cells  $\pm$  S.E. from three independent experiments.



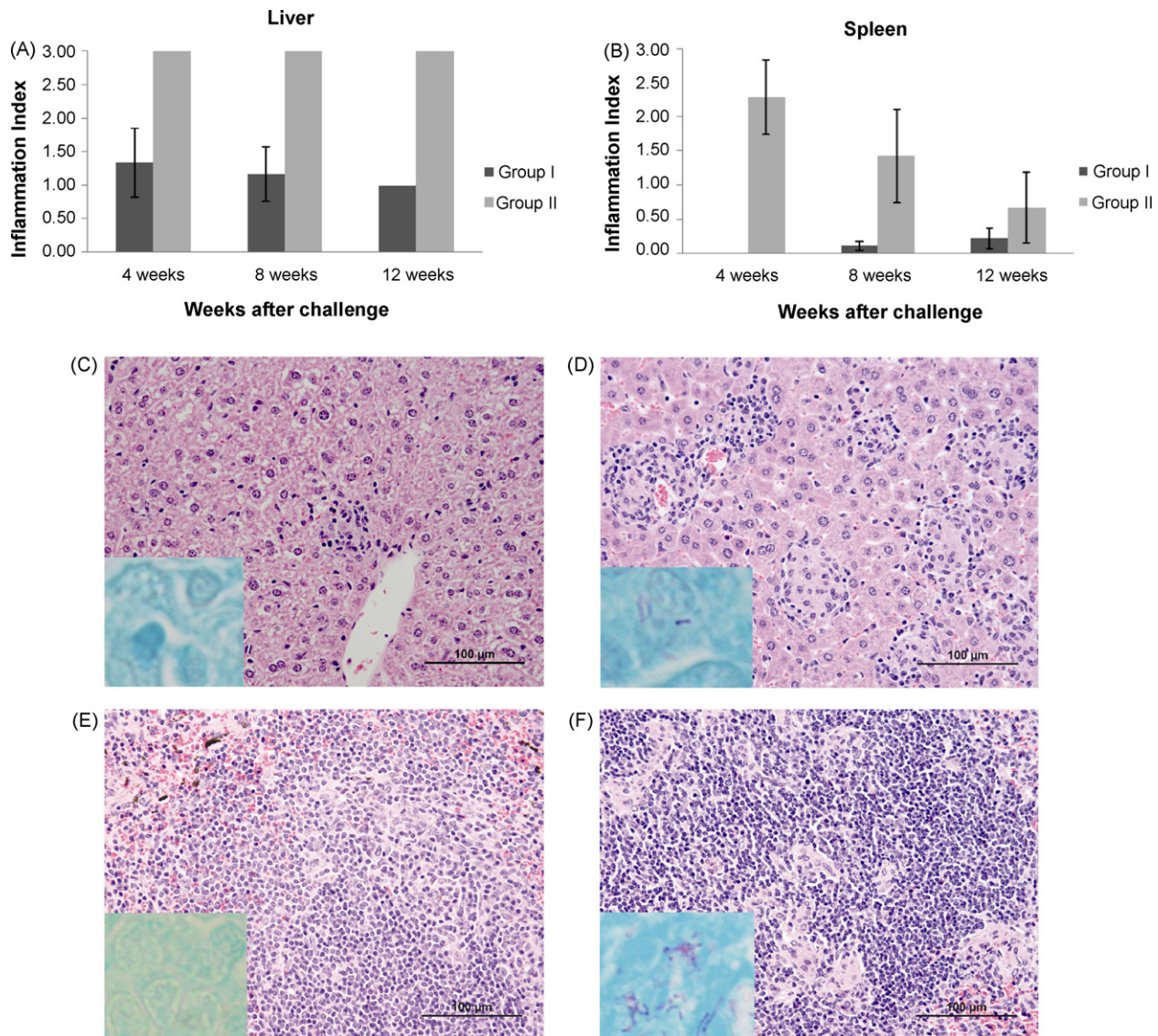
**Fig. 5.** Changes in the mean IgG1 to IgG2a antibody ratio in immunized (group I) and control (group II) mice. Sera were collected from immunized and control animals at 4 weeks after the 2nd booster and checked for antibody response. Data represent mean  $\pm$  S.E. OD value from three independent experiments.

#### 3.5. Immunization with DNA cocktail protects mice against MAP challenge

In order to assess the protective efficacy of the DNA cocktail, histopathological changes and bacterial burden in the liver and spleen after intraperitoneal challenge with MAP were quantified. A significant reduction ( $P < 0.01$ ) in MAP colonization of both the spleen (Fig. 6A) and liver (Fig. 6B) was observed at 4, 8 and 12 weeks post-challenge (PC) in group I animals compared to group II animals. The severity of liver and spleen pathology at 4, 8 and 12 weeks PC paralleled the MAP culture results as distinct differences were noticed between immunized and control animals. MAP challenged group II animals had numerous randomly dispersed granulomas with central epithelioid macrophages surrounded by small



**Fig. 6.** MAP burden in the spleen (A) and liver (B) of mice immunized with either DNA cocktail (group I) or control vector DNA (group II). Mice were infected intraperitoneally with  $10^9$  CFU units of MAP 3 weeks after the 2nd booster immunization. Mice from each group were sacrificed at 4, 8, and 12 weeks after the challenge and the MAP load was enumerated as logCFU/g of organ. Data represent mean  $\pm$  S.E. from three independent experiments.



**Fig. 7.** Histopathological examination of spleen and liver from immunized and control mice. Six mice from each group were sacrificed periodically (4, 8, and 12 weeks after challenge), and tissues sections were stained with hematoxylin and eosin and examined by light microscopy to evaluate granulomatous lesions in spleen (A) and liver (B). The data represent the mean severity score  $\pm$  S.E. (0; no granuloma, 1+, mild, 2++, moderate, 3+++, severe) for each group. (C) Liver from a vaccinated mouse and (D) an unvaccinated control animal. Numerous large granulomas composed almost exclusively of epithelioid macrophages are disseminated throughout the hepatic parenchyma of the unvaccinated mice. A few acid-fast positive organisms are seen within the granulomas (inset). In comparison, only a few small granulomas are found in the livers from vaccinated mice and no acid-fast organisms are noted. (E) Spleen from a vaccinated mouse and (F) an unvaccinated control animal. Numerous granulomas are present in the spleen of the unvaccinated control animals and myriads of acid-fast positive organisms are present (inset). Only rare granulomas are present in the spleen of a vaccinated mouse and no acid-fast organisms are noted.

lymphocytes. Among group I animals, the mean number of granulomas were significantly less ( $P < 0.01$ ) up to 8 week PC in spleen (Fig. 7A) and up to 12 weeks in the liver (Fig. 7B). Ziehl–Neelsen staining of spleen and liver tissue sections revealed numerous acid-fast bacilli in the control animals, whereas the infection was less severe with fewer acid-fast bacilli in animals immunized with the DNA vaccine (Fig. 7C–F). No organisms or granulomas were detected in the lungs or mesenteric lymph nodes (data not shown). No organisms were detected in the feces throughout the study period.

#### 4. Discussion

MAP infection in ruminants causes serious economic losses and, although unconfirmed, may cause Crohn's disease in humans

[35,36]. Several studies have indicated that existing vaccines do not prevent infection in animals and are successful only in limiting the progression of infection and may reduce shedding of the organism. This has resulted in the search for improved vaccines/candidate antigens that can increase vaccine efficacy. Use of DNA vaccines for a wide range of mycobacterial antigens have demonstrated increased protection against various mycobacteria including MAP [37,38]. DNA vaccines typically induce strong CD4<sup>+</sup> and CD8<sup>+</sup> T cell mediated immune responses in mice [37,38]. Because MAP is an intracellular pathogen, cell mediated immunity plays a key role in the control of bacterial replication and subsequent protection against paratuberculosis. In our study, immunization of mice with a DNA cocktail of five MAP genes induced good lymphoproliferative responses. Reportedly, co-immunization using plasmids that encode multiple protective antigens can induce a greater degree

of protection over vaccines consisting of just a single antigen [30,39,40]. Careful selection of antigens used to construct multivalent vaccines can allow for the use of a broad spectrum of MHC molecules in response to the vaccine. When antigen-presenting cells process and present these antigens, they can signal antigen-specific T-lymphocytes in an MHC specific manner, which promotes lymphocyte proliferation. Therefore antigen-specific proliferation can be regarded as an essential feature for selecting potential vaccine candidate antigens for induction of a protective immune response.

Since subsets of T cells are known to be an important factor that affects the efficacy of the host immune response against intracellular organisms [41], we assessed changes in the proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from un-induced and antigen-induced spleen cells. The response of CD4<sup>+</sup> T cells was significantly higher in animals immunized with the DNA cocktail. In one of our earlier studies [28], Ag 85 complex, SOD and 35 kDa were found to stimulate sensitized T cells under *in vitro* conditions in MAP infected cattle. Similarly in the present study, CD4<sup>+</sup> T cells that responded to 85B, SOD, and 35 kDa were significantly higher in DNA cocktail immunized mice. Moreover, *in vitro* stimulation of lymphocytes from control animals given the vector DNA without 85B, SOD, and 35 kDa did not increase the proportion of CD4<sup>+</sup> T cells, indicating that these antigens induce specifically sensitized CD4<sup>+</sup> T cells and may provide protective immunity against MAP. The enhanced CD4<sup>+</sup> T cell response detected against components of the DNA cocktail might have stimulated the production and secretion of cytokines that activate macrophages needed for controlling the infection. However, 85C specific CD4<sup>+</sup> T cell response was less than those evoked by the other antigens, which could be due to low expression levels or differences in the immunogenicity of 85C compared to the other two components of the Ag85 complex [28,42]. The low 85C specific response detected is consistent with the weak expression observed with the 85C DNA construct in western-blot analysis (data not shown).

In mycobacterial infections, apart from limiting the infection, CD4<sup>+</sup> T cells also help in the development of primary CD8<sup>+</sup> T cell responses [43]. CD8<sup>+</sup> T cells likewise, are required for resistance to mycobacterial infections as an alternative source of IFN- $\gamma$ . In our study, all antigens except 85C enhanced the CD4<sup>+</sup> T cell response and also induced higher CD8<sup>+</sup> T cell responses in the immunized animals, which could have led to the increased production of cytokines thereby improving the protective ability of the DNA vaccine. However, additional studies such as intracellular cytokine staining with subset identification are needed to completely exclude bystander effects, which may be caused by the release of cytokines that can non-specifically stimulate proliferation of lymphocyte subpopulations.

We also assessed the type of immune response induced by the DNA cocktail by measuring the expression levels of various cytokines. We used IFN- $\gamma$  levels as a measure of Th1 response as it has been already established by several workers [38,44,45]. Four weeks after the second booster immunization, IFN- $\gamma$  gene expression levels were higher in immunized animals compared to the controls. The results of ELISPOT assay also revealed an increase in the number of IFN- $\gamma$  secreting cells in the immunized group relative to the control group, indicating a Th1 type response. A T cell mediated Th1 response, rather than a Th2 cell mediated antibody response, is considered to play a key role in resisting MAP infections [46]. In contrast to the expression of Th1 cytokines, expression of Th2 cytokines such as IL-4 and IL-10 were low in the immunized group. Overall, these results show an increase in the Th1 response rather than a Th2 response. Vaccination with plasmids encoding mycobacterial genes generates cell-mediated, humoral and protec-

tive immune responses [20,38,47–49]. In our study, immunization with the DNA cocktail generated protective immunity in mice as demonstrated by reduced MAP burden in the spleen and liver. This was further confirmed by the liver and spleen pathology in terms of the number of granulomas and acid-fast bacilli. DNA vaccinated animals resisted MAP challenge showing fewer granulomas and acid-fast bacilli compared to the control animals demonstrating the protective nature of the DNA vaccine. However, the levels of protective immunity gradually decreased in the DNA vaccinated groups when we compared the results at each time period. These results are very similar with previous findings that levels of protective immunity decrease between 4 and 8 weeks after challenge [50,51].

DNA vaccination, with naked bacterial plasmid DNA, is a relatively easy and safe method to induce strong humoral and cellular immune responses [51]. DNA vaccines induce strong CD4<sup>+</sup> and CD8<sup>+</sup> mediated immune responses in mice [21]. MAP is an intracellular pathogen, therefore, cell mediated immunity plays a key role in the control of the bacterial replication and the subsequent protection against paratuberculosis [52]. Not all studies have analyzed the immune responses in parallel with protection, but high, antigen-specific pre-challenge spleen cell IFN- $\gamma$  levels often correlate with the best protection [21]. In our study, we observed high levels of antigen specific IFN- $\gamma$  levels in spleen cells indicative of protection. Secreted and surface-exposed cell wall proteins are major antigens recognized by the protective immune response against MAP infection. A major fraction of the secreted proteins in MAP culture filtrate is formed by the Ag85 complex, a 30- to 32-kD family of three proteins (Ag85A, Ag85B, and Ag85C) [53]. Superoxide dismutase of MAP protects the organism against oxidative stress by catalyzing the dismutation of superoxide radicals and has also been reported to induce a strong Th1 response in a mouse model [54]. Although less immunostimulatory than other antigens, SOD also strongly induced  $\gamma\delta$ <sup>+</sup> T cells, which are thought to be important in the early stages of infection [41]. Therefore we included SOD in our vaccine cocktail in order to enhance the vaccine efficacy. 35 kDa protein is identified as a potential virulence factor [55]. In the present study, all antigens except 85C were strongly recognized by the mouse immune system. The weak recognition of Ag85C molecule in our study might be caused by lower expression levels in the host system rather than by lower immunogenicity of the molecule. The 85C molecule was also reported to have a lower secretion index than the Ag85A and Ag85B molecule [56]. It is evident from our study that, immunization with the DNA cocktail induced modest levels of protective immunity against MAP challenge. The major limitation of studying a cocktail vaccine is that it is impossible to assess which immunogen is most important for protection. However, it has been reported that vaccination with either a DNA vaccine cocktail or a multicomponent subunit vaccine induces better protection against challenge by *M. tuberculosis* [20,39,57,58]. Careful selection of antigens used to construct multivalent vaccines can allow for the use of a broad spectrum of MHC molecules in response to the vaccine. In conclusion, our data also indicate that Ag85 complex, SOD and 35 kDa are promising candidates for developing a DNA vaccine for paratuberculosis in ruminants.

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